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Our long-term objectives are to define the molecular processes contributing to lung cancer development and progression in order to recognize genetic and phenotypic changes early enough to be reversed with molecularly-targeted therapy and to develop innovative therapeutic approaches to lung cancer. Therefore, the specific goals of this program are to understand molecular alterations in lung cancer, develop lung cancer prevention strategies, and implement experimental molecular approaches to lung cancer. We report herein that aberrant splicing of DNMT3B6 may be involved in tumorigenesis and epigenetic alteration events; anti-tumor effects of COX-2 inhibitors are not dependent on COX-2 expression and are significantly enhanced by retinoic acid; genetic inhibition of the intracellular signaling molecule phosphatidyl inositol 3'-kinase results in induction of apoptosis in lung cancer cell lines; the newly-identified FUS1 gene acts as a potent tumor suppressor in animal models without cytotoxic effects; intravenous injection of the GFE1 peptide results in distribution to pulmonary arteries, veins, bronchial arteries and alveolar capillaries, and reduces the number and size of pulmonary metastases in an animal model of metastatic lung cancer.

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INTRODUCTION

Lung cancer is a devastating worldwide public health hazard, with a total of 1,500,000 new cases anticipated in the year 2000 (1). Advances in the surgery, radiation therapy, and chemotherapy of non-small cell lung cancer have led to an improvement in five-year survival from 7% in 1970 to 15.8% IN 1994 (2). To reduce lung cancer incidence and mortality, it is imperative to develop effective therapeutic and preventive strategies targeting smokers and lung cancer patients. Our long-term objectives are to define the molecular processes contributing to lung cancer development and progression in order to recognize genetic and phenotypic changes early enough to be reversed with molecularly-targeted therapy and to develop innovative therapeutic approaches to lung cancer. The objectives can be met only by understanding the biology of lung cancer through molecular studies and preclinical experimental molecular therapeutic research. Therefore, the specific goals of this program are to understand molecular alterations in lung cancer, develop lung cancer prevention strategies, and implement experimental molecular approaches to lung cancer.

PROGRESS REPORT

Specific Aim 1 Mechanisms of Molecular Alterations in Lung Cancer

Specific Aim 1.1 Determine the mRNA splicing complex responsible for C-CAM splicing and identify potential altered component(s) in lung cancer cells

To analyze mechanisms of alternative splicing of C-CAM1, we constructed a mini-gene model with pCMV vector containing a DNA fragment from intron 5 to intron 8 of the C-CAM1 gene. When the vector is transfected into cancer cell lines, we can replicate alternative splicing phenotypes consistent with the native splicing patterns of C-CAM1. It provides a good instrument for further studying splicing control mechanisms.

By analyzing several constructs with different deletions of part of the mini-gene, we found that part of the exon 7 sequence itself is essential for inclusion and exclusion of the exon. We further conducted two experiments to identify potential splicing-related proteins essential for C-CAM1 splicing variants. We first extracted nuclear proteins from four cancer cell lines; two cell lines express predominantly the L-form-C-CAM1 (H1944 and A549) and two express predominantly the S-form-C-CAM1 (H460 and 17B). We found differential protein expression between the two groups of cell lines. A group of proteins at pH 10 and about 40kD in size were highly-expressed in H1944 and A549 but not in H460 and 17B. In contrast, several lower molecular weight proteins were expressed in the H460 and 17B cell lines. We analyzed the differentially-expressed proteins and found most of the proteins are hnRNPs consisting of various isoforms and alternatively spliced forms. These results suggest that hnRNPs and their alternatively spliced forms may play important roles in controlling C-CAM1 splicing patterns.

We then performed sequence-specific affinity chromatography using a specific exon 7 sequence to isolate proteins binding specifically to the sequence. The experiment yielded two specific proteins. We analyzed the proteins by peptide digestion and mass spectrometry. The two proteins turned out to be hnRNP A1 and polypyrimidine tract-binding protein (PTB) both known involved in splicing regulation.

Specific Aim 1.2 ***Determine functions of the identified component(s) in pre-mRNA splicing control of C-CAM and potential alterations in lung cancers***

These studies are planned for Years 3-5 of the granting period.

Specific Aim 1.3 ***Determine the function of DNA methyltransferases and their genes in controlling methylation and the expression status of critical tumor supressor genes and tumor antigen genes***

We have discovered a novel promoter (DNMT3B6) of the DNMT3B gene. We have found that DNMT3B6 is the predominant form of DNMT3B In different epithelial cells, white blood cells and individual samples.

A T-C transition mutation was found in upstream of DNMT3B6. This mutation located at 286bp upstream of the DNMT3B6 transcription start site and could change a TFIID (CTcTATTCCA) binding site to GATA-1 (TCTAT**C**) binding site. Interestingly, this T-C mutation is coupled with another C-T transition polymorphism located 147bp upstream of the DNMT3B6 transcription start site; this mutation allows easy detection of genotype distribution of a large population. In a small case-control study in lung cancer patients, we found that this polymorphism shows strong association with higher risk in lung cancer patients. Another large-scale case control investigation has been conducted and we found that compared with the CC genotype, the CT heterozygotes were associated with more than two-fold increased risk [adjusted odds ratio (OR) = 2.13; 95% confidence interval (CI) = 1.47-3.08]. A manuscript describing these results has been submitted.

The mRNA expression profile of DNMT3B6s in normal lung cDNA library and NSCLC cell lines has been evaluated using RT-PCR. At least seven new isoforms were detected; the isoforms reflect difference inclusionand exclusion patterns of exons 7, 8, 9 and 10. These seven isoforms of the DNMT3B6 family have been named DNMT3B6s subfamily (3B6-a, -b, -c, -d, –e, -f and –g). The result from RT-PCR also showed that expression of these DNMT3B6 transcripts is variable in different cell lines and primary NSCLC samples. Interestingly, comparative analysis of putative amino acid sequences shows an association with an undefined PWWP domain; PWWP domain is a proline-tryptophan-tryptophan-proline motif in the consensus amino acid sequence (ProDom database, ID14260). We suspect that this domain will be strongly associated with the substrate specificity of DNMT3B6 and the further research on this part is ongoing.

Specific Aim 1.4 ***Determine expression profiles and abnormalities of DNMT3B isoforms in lung tumorigenesis and their association with de novo DNA methylation patterns, as well as potential clinical applications***

These studies are planned for Years 4 and 5 of the granting period.

Specific Aim 2 Novel Strategies for Lung Cancer Chemoprevention

Specific Aim 2.1 ***Evaluate the effects of aerosolized 13cRA delivered to former smoker by inhalation alone or in combination with Celecoxib***

This clinical trial is not scheduled to be initiated until Year 3 of the granting period.

Specific Aim 2.2 ***Evaluate the effects of NSAIDS and 13cRA as single agents and in combinations on growth, apoptosis and carcinogenesis using an in vitro cell system and an animal model***

One approach to prevent lung cancer and suppress the field of cancerization in the aerodigestive tract is the use of retinoids to modulate growth and apoptosis of premalignant and malignant epithelial cells. Retinoids are critical for maintenance of respiratory epithelial cell differentiation and can induce terminal differentiation or apoptosis in initiated epithelial cells. There are, however, several drawbacks to the use of these agents. Among these is a profile of toxic effects that can render long-term therapy difficult to tolerate. If the therapeutic benefit can only be maintained with long-term therapy, the issue of patient compliance is pivotal. Therefore, we propose to investigate the potential of combining lower doses of retinoic acid with non-steroidal anti-inflammatory agents. The ability of various non-steroidal anti-inflammatory agents to suppress carcinogenesis in animal models indicates the potential clinical application. This potential has been demonstrated in humans, especially in the prevention of gastrointestinal cancers. However, it is not known whether non-steroidal anti-inflammatory agents could also be useful for preventing lung cancer.

Inhibitors of COX-2 and 5-LOX have shown interesting effects on growth and apoptosis of various tumor cell lines, including non-small cell lung cancer (NSCLC) cells. Thus, we are evaluating the activity of COX-2 and 5-LOX inhibitors used as single agents and in combination with each other and with retinoic acid in suppression of cell growth and induction of apoptosis in normal, immortalized, premalignant, transformed and tumorigenic lung cancer cells lines and to relate these effects to COX-2 expression. Emphasis will be given to analysis of changes in cell cycle and apoptosis related genes. A/J mice will be exposed to the tobacco carcinogen NNK and treated with a selected COX-2 and 5-LOX inhibitor and retinoic acid used as single agents and in combination to determine their efficacy to decrease the incidence of lung adenomas and adenocarcinomas. The mechanisms involved in the *in vivo* effects will be examined based on the results of the *in vitro* studies.

We have conducted studies to evaluate the effect of tissue culture conditions on expression of COX-2 in normal, premalignant and malignant lung epithelial cell lines. Preliminary results indicate that normal bronchial epithelial cells in submerged short-term monolayer cultures express COX-2 protein and that premalignant cells expressed lower COX-2 levels. Among non-small cell lung cancer cell lines, some expressed COX-2 and others did not. These findings did not support the contention that was based on findings in other tumor types and lung tissue *analysis in vivo*, namely that normal cells express low or no COX-2, whereas tumor cells do express this enzyme. Therefore, we investigated this issue further. We addressed two questions, the first was whether the way we cultured the normal human bronchial epithelial (NHBE) cells affected COX-2 expression. The NHBE cells obtained from Whittaker/Clonetics are grown in keratinocyte growth medium produced and recommended by the manufacturer. This is a serum-free medium and it supports the growth of NHBE cells in submerged monolayer cultures. However, under these conditions, the cells do not form cilia and do not produce mucin glycoproteins (3). Interestingly, if the same cells are cultured on collagen gels at the air:medium interphase, then the cells form ciliated cells with goblet-like cells that produce mucin. Thus cells grown in such organotypic three-dimensional raft cultures form a mucocilliary epithelium, which resembles the *in vivo* differentiation of bronchial epithelial cells (4). In collaboration with Dr. Peter Koo at this institution, we found that COX-2 expression was high in submerged NHBE cultures and decreased markedly and progressively after 2 and 8 weeks of growth in raft cultures. The finding that some NSCLC cell lines (e.g., H358 and A549) expressed COX-2, whereas others (e.g., H460, H1792) failed to express the enzyme was perplexing because the

majority of lung tumors *in vivo* do express COX-2. Given that NHBE cells expressed COX-2 when cultured in serum-free medium whereas the NSCLC cells were grown in serum-containing medium, we asked whether the presence of serum in the growth medium of NSCLC might affect expression COX-2. We found that the removal of serum from the growth medium did not increase COX-2 expression in NSCLC cells that failed to express this enzyme in serum-containing medium and partially decreased COX-2 levels in cells that did express COX-2 in the presence of serum. It is possible that COX-2 suppression in a subset of NSCLC cell lines is caused by methylation of CpG islands in the COX-2 gene (5) rather than by the presence or absence of growth factors in the medium.

We have conducted studies to determine if there is a correlation between expression of COX-2 and response to non-steroidal anti-inflammatory agents. Preliminary results indicate that the growth inhibitory effects of the NSAID NS398 were not related to the expression of COX-2. Thus, NS398 inhibited NSCLC cell lines H358 and A549, which express COX-2, with an IC_{50} of 76 and 100 μM, respectively and cell lines H1792 or H460, which do not express COX-2, with IC_{50} of 95 and 75 μM, respectively. We now extended these studies to the COX-2 inhibitor Celecoxib, which has been approved by the Food and Drug Administration for use in prevention of colon cancer in high-risk population. We found that the IC_{50} of this selective COX-2 inhibitor in the BEAS-2B premalignant cell line and several NSCLC cell lines (including some which express COX-2 and some which do not) were in the range of 17 and 36 μM. Thus, there does not appear to be a relationship between COX-2 expression and response. The finding confirms a recent report that fibroblasts from COX-2 knockout mice were as sensitive to Celecoxib as fibroblasts from the wild-type mouse (6).

We have begun to evaluate the effects of combinations of retinoic acid and NS398 on growth of premalignant and malignant lung epithelial cell lines. Preliminary results indicate that the COX-2 inhibitor NS398 and the 5-LOX inhibitor MK886 suppress the growth of premalignant and malignant lung epithelial cell lines. We have extended these studies to determine whether combinations of each of these agents with retinoic acid will have better growth inhibitory effects than each agent alone. We found that treatment with the combinations of retinoic acid with either of these agents resulted in less than additive effects on cell growth in some cells and in no increase in effect relative to single agent activity in other cells. In no case did we detect synergistic effects.

Specific Aim 2.3 Investigate whether genetic approaches to inhibit PI3K activity decrease lung tumor size and number in k-ras mutant mice

Growth factor-induced activation of phosphatidylinositol 3-kinase (PI3K) promotes cancer cell survival. We have found that inhibition of the catalytic activity of PI3K with either PTEN or LY294002 induced proliferative arrest but not apoptosis of lung cancer cells. In contrast, transfection of a dominant-negative mutant of the p85α regulatory subunit of PI3K (Δp85) induced apoptosis. PTEN and LY294002 effectively inhibited PI3K-dependent signaling, whereas Δp85 inhibited signaling through both PI3K- and RAC-1-dependent signaling cascades. A constitutively-active mutant (V^{12}) Rac-1 abrogated Δp85-induced lung cancer cell death. Inhibition of RAC-1-dependent signaling enhanced cell death induced by LY294002. We found that direct interactions between RAC-1 and p85α were required for RAC-1 activation by peptide growth factors. We propose that p85α promotes lung cancer cell survival through activation of PI3K- and RAC-1-dependent pathways.

Specific Aim 3 Experimental Molecular Therapeutic Approaches to Lung Cancer

Specific Aim 3.1 *Develop a relatively faithful murine model of lung cancer by crossing the k-ras mutant mouse with our p53 mutant mis-sense mouse so that we can study the evolution of non-small cell lung cancer in a primary lung tumor model with metastatic potential, as well as the effectiveness of molecularly targeted strategies*

The first experiments were set up to obtain mice with both mutations in an inbred background. The *p53R172HΔg+/-* mice are in a mixed 129Sv and C57BL6/J background and have been backcrossed to 129Sv mice to create a pure strain. Three generations have been back-crossed (each generation takes approximately 3 months) and the mice are presently greater than 90% 129Sv. This is an important first step because it has generated an inbred strain of mice, thereby eliminating the role of genetic variation in development of tumors.

The *ras* mice are already in the 129Sv background but needed to be embryo-derived into a Specific Pathogen Free (SPF) facility. This task has been accomplished.

The assays for genotyping mice are in place. Both mice have reached reproductive age and are being crossed to each other to establish the following cohort: *p53R172HΔg+/-*, *ras +/-*, and *p53R172HΔg+/- ras +/-*. Approximately 50 mice for each of the genotypes will be generated. We have found that analysis of this number of mice yields statistically relevant data.

Specific Aim 3.2 *Evaluate novel signal transduction inhibitors, both alone and in combination with one another and with cytotoxic agents, in the treatment of mouse lung cancer models and ultimately in the treatment of human lung cancers*

Farnesyl transferase inhibitors are a class of compounds developed to inhibit the *ras* oncogene. Several studies have shown them to have cytotoxic and cytostatic effects in preclinical models of lung cancer (7,8). R115777 was the first farnesyl transferase inhibitor to enter clinical trials (9). It is being studied in combination with different cytotoxic agents in the therapy of non-small cell lung cancer. We are interested in testing this agent in the reversal of lung premalignant phases, as well as in combination with novel cytotoxics.

Agents such as ZD1839, aimed at inhibiting signal transduction mediated through the epidermal growth factor receptor (EGFR), are currently under clinical investigation (10). ZD1839 is a potent and specific inhibitor of EGFR kinase, and its use in epithelially-derived EGFR-expressing and over-expressing NSCLC is grounded in strong biological rationale. Phase I trials have now confirmed appreciable anti-tumor activity of ZD1839 when used as monotherapy (41). ZD1839 also appears to potentiate the anti-tumor and apoptotic effects of a number of cytotoxic agents in human tumor xenograft models, including models of NSCLC (11,12).

We have evaluated the effects of SCH66336, an oral farnesyl transferase inhibitor, on growth and apoptosis in the NSCLC cell line A549. The first experiments show that at doses of 1.5 mg, SCH66336 inhibited A549 cell growth. The growth inhibitory effects appear to be largely due to cytotoxic effects on the cells; in these experiments, very little induction of apoptosis was detected. Further, SCH66336 induced down-regulation of phosphorylated raf, and decreased the levels of both total cellular Akt and phosphorylated Akt. Down-regulation of total cellular Akt

by farnesyl transferase inhibitor is a new finding and we are currently studying the mechanism of this effect.

Furthermore, we have generated head and neck and lung cancer cell lines with and without h-ras and k-ras mutations, and are in the process of evaluating the efficacy of SCH66336 in the context of ras mutation status. Preliminary data demonstrate no relationship between sensitivity to the cytotoxic and cell cycle effects of SCH66336 and h-ras mutational status.

We are beginning experiments to study the effects of combining SCH66336 with ZD1839 in both NSCLC and head and neck cancer cell lines. We have also begun evaluating the combination of 4-HPR (fenretinide), a pro-apoptotic retinoid, with SCH66336. We have observed enhancement of apoptosis; while the data are preliminary, there is a suggestion that the combination may have synergistic activity. We are conducting similar experiments with the SCH66336 and ZD1839 combination.

Specific Aim 3.3 ***Produce and test a liposomal gene-therapeutic strategy targeted to a novel tumor suppressor gene located on chromosome 3p, both in the mouse model and in human patients with advanced non-small cell lung cancer***

Genetic alterations and allelic losses on the short arm of chromosome 3 (3p) are the most frequent and earliest cancer abnormalities detected in human lung cancers. The novel FUS1 gene is one of the candidate tumor suppressor genes identified in a 120-kb homozygous-deletion region in human chromosome 3p21.3. We have used recombinant adenoviral vectors (Ad-FUS1) or DOTAP-Cholesterol-liposome-complexed FUS-containing plasmid DNA (FUS1-lipoplex) to introduce wild-type FUS1 gene into 3p21.3 gene-deficient human NSCLC cells to evaluate *in vitro* and *in vivo* tumor-suppressing activity. We found that ectopic expression of FUS1 by either adenoviral vector or lipoplex-mediated gene transfer effectively inhibited tumor cell growth by altering cell cycle processes and inducing apoptosis *in vitro* and *in vivo*. These findings strongly suggested the role of the FUS1 gene as a tumor suppressor gene.

To test the hypothesis that the wild-type FUS1 gene functions as a tumor suppressor gene, we studied the effects of FUS1 expression on cell proliferation in several types of Ad-FUS1-transduced human NSCLC cells and a normal human breast epithelial cell (HBEC). We fond that cell viability was significantly reduced in Ad-FUS1-transduced A549 cells, which are homozygous for multiple 3p21.3 markers and contain wild-type p53, and in H1299 cells, which are homozygous for both 3p21.3 markers and for deletion of p53. However, no significant effects on growth were observed in any cells transduced with control vectors. These results suggest that exogenous expression of wild-type FUS1 either inhibited 3p-deficient tumor cell growth or restored the tumor suppressor function of the FUS1 gene. To clarify the specificity of the observed effects on tumor cell growth and to examine the cytotoxicity of the exogenously expressed FUS1 on normal cells, we determined the effects of the FUS1 gene on cell proliferation in 3p21.3-heterozygous H358 cells and normal HBECs. Both HBECs and H358 cells transduced with Ad-FUS1 exhibited a loss in cell viability of less than 10% as compared with the untransduced control cells. Similar levels of loss were observed in H358 cells and HBECs transduced with Ad-EV and Ad-LacZ. These results, coupled with the lack of effect with Ad-LacZ and Ad-HYAL, demonstrate the specificity of the tumor-suppressing function of the FUS1 gene in 3p-deficient tumor cells and indicate that no generalized cytotoxicity is associated with exogenous expression of the wild-type FUS1 gene.

We analyzed the ability of exogenously-expressed FUS1 to induce apoptosis in the Ad-FUS1-transduced H1299, A549 and H358 lung cancer cell lines and HBEC cells. We fond that apoptosis was induced in Ad-FUS1-transduced A549 and H1299 cells, but not in H358 and HBECs. The levels of apoptosis and the timecourse of its induction in cells transduced by Ad-FUS1 correlated with the levels of inhibition of tumor cell growth in other cells treated with the same vectors, suggesting that suppression of tumor cell proliferation by FUS1 was mediated directly or indirectly through a mechanism of apoptosis induction.

To determine whether the observed inhibitory effects of FUS1 on *in vitro* cell proliferation would have *in vivo* relevance, we evaluated the efficacy of FUS1 in suppressing tumor growth by direct intratumoral injection of Ad-3p21.3 vectors, along with PBS and Ad-EV, Ad-LacZ and Ad-p53 vectors as controls, into A549 or H1299 tumor xenografts in nu/nu mice. The growth of tumors was recorded from the day of the first injection until 20 days after the last injection. Ad-FUS1 significantly suppressed tumor growth in both A549 and H1299 tumor models as compared with mouse models treated with Ad-LacZ or Ad-EV controls.

To explore the potential of FUS1 gene to suppress systemic metastases, we developed a novel formulation using protamine-adenovirus (P-Ad) complexes. For these studies, we used the experimental A549 metastatic human lung cancer model in nu/nu mice and evaluated effects on development of lung metastases. The Ad-3p21.3 gene vectors were complexed to protamine and delivered via intravenous injection. A549 metastases were significantly inhibited; the formation of metastatic tumor colonies on the surfaces of lungs from mice inoculated with A549 cells was reduced more than 80% in animals treated with P-Ad-FUS1 as compared with those treated with controls. However, metastatic colony formation was not significantly reduced in animals treated with P-Ad-HYAL1 control vector. These data were consistent with results obtained from Ad-FUS1-treated subcutaneous tumors, further supporting the role of FUS1 in suppressing tumor growth and inhibiting tumor progression *in vivo*.

On the basis of studies describing efficient systemic delivery using a DOTAP:cholesterol lipid formulation, we have conducted preliminary studies using a DOTAP:cholesterol lipid formulation and have demonstrated the feasibility of *in vivo* gene delivery via systemic routes. Liposomes (20 mM DOTAP:cholesterol) were synthesized as described (13) and mixed with β-gal plasmid DNA to give a final concentration of 150 mg of liposomes per 300 mL of β-gal-lipoplex. A single 50 mg dose of lipoplex was intravenously injected into immunocompetent female C3H mice via the tail vein, and mice were monitored for toxicity, as determined by mortality and morbidity rates. Toxicity also was assessed through measurement of liver enzyme profiles 24 and 48 hours after injection. No animals died in the study, suggesting no overt toxicity. Liver enzyme profiles (OT/PT and AP) showed no significant differences in enzyme levels between treated and untreated animals.

We have conducted preliminary studies to determine the extent of gene transfer and subsequent expression levels of a β-gal reporter plasmid transfer via DOTAP:cholesterol liposomes. When administered by intralesion injection into subcutaneous tumors formed from human H1299 lung cancer cells, 200 mL of β-gal-lipoplex (100 mg) caused marked gene expression 48 hours after injection. A transfection efficiency of 25-30% was observed upon a single intratumoral injection. Similarly, extruded DOTAP:cholesterol liposomes were compared to both non-extruded DOTAP: cholesterol liposomes and another conventional liposome formulation (DOTAP:DOPE) in a therapeutic xenograft model of human lung metastasis using H1299 and A549 human lung cancer cells. Analysis of lungs from SCID/beige mice bearing p53-null H1299 lung tumors revealed significantly fewer experimental metastases in mice

injected via the tail vein with an extruded DOTAP:cholesterol-p53 complex than in any of the control groups. These results suggested nonviral nucleic acid delivery as a viable cancer treatment approach.

Specific Aim 3.4 ***Develop specific vascularly targeted strategies to the vascular endothelium of lung cancer cells in order to decrease the toxicity to normal cells and enhance the therapeutic index.***

In vivo phage display is a powerful technique for identifying oligopeptide sequences capable of binding to specific target proteins or cells. Random peptide phage libraries injected intravascularly have been able to identify unique peptide sequences capable of binding to "vascular addresses" on different organs and tissues (14). This technique applied in a mouse model has previously identified peptides with the "GFE" motif as selective for the pulmonary vasculature. The receptor for these lung-homing GFE peptides has been identified as membrane dipeptidase (MDP, also known as renal dipeptidase or microsomal dipeptidase). MDP is also known as the renal tubular enzyme responsible for cleaving and inactivating the antibiotic imipenem (15). GFE peptides appear to inactivate MDP enzymatic activity.

This interaction of GFE and MDP in the lung appears to be very relevant in the development of lung malignancies. The phage clone bearing the GFE peptide (CGFECVRQCPERC) was re-injected into mice bearing melanoma lung metastases. As expected, these phage could be recovered only from normal lung, but not from melanoma metastases. Surprisingly, however, intravenous injection of the GFE peptide was able to reduce the number and size of pulmonary metastases in this mouse model of lung metastases.

Preliminary *in situ* experiments show that rhodamine-labelled GFE peptide binds in human lung as well, distributed in pulmonary arteries, veins, bronchial arteries and alveolar capillaries. The binding of GFE rhodamine to these structures is specific and can be blocked by unlabelled CGFEC peptide. Currently, GFE peptide is being evaluated for its distribution in tumor-bearing lung and for its selectivity for binding to lung vasculature.

If lung tumor vasculature shows an absence of staining with labeled GFE as predicted by mouse experiments, GFE might be useful for prevention of development of lung metastases or primary lung malignancies. Furthermore, if the mechanism of inhibiting lung metastases is related to inactivation of MDP, pharmaceuticals such as cilastatin (a co-drug used to inhibit degradation of the antibiotic imipenem) may be equally effective at inhibiting development of lung metastases. If the above data are convincing, a pilot trial of cilastatin or similar drugs might be attempted in melanoma or sarcoma patients with early lung metastases or with high risk of lung metastases. Additional animal experiments might also help determine whether GFE or MDP inhibitors might help prevent the development of primary lung cancers.

KEY RESEARCH ACCOMPLISHMENTS

- We have shown that part of the exon 7 sequence itself is essential for inclusion and exclusion of exon 7 in C-CAM.
- hnRNPs and their alternatively spliced forms may play important roles in controlling C-CAM1 splicing patterns. hnRNP A1 and polypyrimidine tract-binding protein (PTB) were found to be associated with exon 7 of C-CAM.

- A number of new isoforms of DNMT3B have been detected. Some isoforms are present in lung cancer, including cell lines and clinic samples, suggesting that aberrant splicing of DNMT3B6 may be involved in tumorigenesis and epigenetic alteration events.
- COX-2 expression is high in NHBE cells grown in submerged cultures where they do not express mucocilliary epithelium, but low in NHBE cells which are grown in organotypic cultures where they do express the mucocilliary epithelium. Thus, the organotypic cultures are more representative of normal bronchial epithelial cells for studies of COX-2 expression.
- NSCLC cell lines that express COX-2 do not show greater sensitivity to COX-2 inhibitors than those that do not express COX-2.
- The combination of retinoic acid with either the COX-2 inhibitor NS398 or the 5-LOX inhibitor MK886 is not more effective than the sum of the activities of each agent alone and in some cases is not better than single agent alone.
- Inhibition of the catalytic activity of PI3K with either PTEN or LY294002 induces proliferative arrest but not apoptosis of lung cancer cells.
- Transfection of a dominant-negative mutant of the p85α regulatory subunit of PI3K (Δp85) induces apoptosis.
- PTEN and LY294002 effectively inhibit PI3K-dependent signaling, whereas Δp85 inhibits signaling through both PI3K- and RAC-1-dependent signaling cascades.
- A constitutively-active mutant (V^{12}) Rac-1 abrogates Δp85-induced lung cancer cell death. Direct interactions between RAC-1 and p85α are required for RAC-1 activation by peptide growth factors.
- The first experiments were set up to obtain mice with both mutations in an inbred background. The *p53R172HΔg+/-* mice are in a mixed 129Sv and C57BL6/J background and have been backcrossed to 129Sv mice to create a pure strain. Three generations have been back-crossed.
- SCH66336 appears to have substantial activity against various non small cell lung cancer cell lines assay, regardless of k-ras mutational status or h-ras mutational status.
- The combination of SCH66336 and 4-HPR enhances apoptosis.
- We found that cell viability was significantly reduced in Ad-FUS1-transduced A549 cells, which are homozygous for multiple 3p21.3 markers and contain wild-type p53, and for H1299 cells, which are homozygous both for 3p21.3 markers and for deletion of p53.
- We demonstrated the specificity of the tumor-suppressing function of the FUS1 gene in 3p-deficient tumor cells and indicated that no generalized cytotoxicity was associated with exogenous expression of the wild-type FUS1 gene.
- Ad-FUS1 significantly suppressed tumor growth in both A549 and H1299 tumor models, when compared with mouse models treated with Ad-LacZ or Ad-EV controls.
- Preliminary *in situ* experiments show that rhodamine-labelled GFE1 peptide binds in human lung as well, distributed in pulmonary arteries, veins, bronchial arteries and alveolar capillaries.
- The binding of GFE rhodamine to these structures is specific and can be blocked by the presence of unlabelled CGFEC peptide.
- Intravenous injection of the GFE1 peptide was able to reduce the number and size of pulmonary metastases in this mouse model of lung metastases.

REPORTABLE OUTCOMES

- Soria JC, Lee HY, Lee JI, Wang L, Issa JP, Kemp BL, Liu DD, Kurie JM, Mao L, Khuri FR. Lack of PTEN expression in NSCLC is related to promoter methylation. (in press, *Clinical Cancer Research*).
- Ho-Young Lee, Harish Srinivas, Yiling Lu, Robert Superty, Ruth LaPushin, Candelaria Gomez-Manzano, Anna Maria Gal, Garrett L. Walsh, Thomas Force, Gordon B. Mills and Jonathan M. Kurie. The p85α Regulatory Subunit of Phosphatidylinositol 3-kinase (PI3K) Stimulates Lung Cancer Cell Survival through Activation of PI3K- and RAC-1-dependent Pathways (submitted for publication, *Molecular and Cellular Biology*).
- Hongbing Shen, Luo Wang, Margaret R. Spitz, Waun K. Hong, Li Mao and Qingyi Wei. A Genetic Variant of *DNMT3B* Promoter and Risk of Lung Cancer – A Case-Control Study (submitted for publication, *Cancer Research*).
- Luo Wang, Shiyong Sun, Marivonne Rodriguez, Ping Yue, Weiguo Wu, Reuben Lotan, Waun Ki Hong and Li Mao. Alternative and Aberrant Splicing of Cytosine-DNA Methyltransferase 3B (DNMT-3B) (in preparation).

CONCLUSIONS

Specific Aim 1 DNMT3B structure at the transcriptional and post-transcriptional level is quite complex and a number of new isoforms have been detected. Some isoforms are present in lung cancer, including cell lines and clinic samples, suggesting that aberrant splicing of DNMT3B6 may be involved in tumorigenesis and epigenetic alteration events.

Specific Aim 2 The mechanism of action of NSAIDs on normal premalignant and malignant lung epithelial cells may be independent of COX-2 inhibition. The combination of retinoic acid and either NS398 or MK886 does not appear to have advantage over single agent.

Studies conducted with the PI3K inhibitors PTEN and LY294002 show that these agents induce proliferative arrest but not apoptosis of lung cancer cells. In contrast, genetic inhibition of the p85α regulatory subunit of PI3K induced apoptosis and inhibited signaling through both PI3K- and RAC-1-dependent signaling cascades. These data support the hypothesis that p85α promotes lung cancer cell survival through activation of PI3K- and RAC-1-dependent pathways.

Specific Aim 3 We have begun the initial experiments crossing a k-ras stochastic murine model of lung cancer and a p53 mis-sense mutation mouse model of lung cancer. We have started to assay in preclinical systems the differential activities of SCH66336 and ZD1839. We have made major accomplishments towards development of a novel gene therapy approach utilizing a FUS-1 gene and have started animal experiments in this regard. We have also completed substantial work focused on vascular targeting of lung cancer and utilizing *in situ* data demonstrating that rhodamine-labeled GFE peptide lines are found throughout the human lung vasculature.

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